

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
18 December 2003 (18.12.2003)

PCT

(10) International Publication Number
WO 03/103737 A1

(51) International Patent Classification⁷: **A61L 27/22, 27/46, 27/56, C08H 1/06, C08L 89/04, C08K 3/32**

(21) International Application Number: **PCT/NZ03/00116**

(22) International Filing Date: **10 June 2003 (10.06.2003)**

(25) Filing Language: **English**

(26) Publication Language: **English**

(30) Priority Data:
519456 10 June 2002 (10.06.2002) NZ

(71) Applicants (*for all designated States except US*): **WOOL RESEARCH ORGANISATION OF NEW ZEALAND (INC) [NZ/NZ]; Cnr Springs Road & Gerald Street, Lincoln, Christchurch (NZ). UNIVERSITY OF OTAGO [NZ/NZ]; Leith Street, Dunedin (NZ).**

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **PEPLOW, Philip, Victor [GB/NZ]; 17 Garfield Avenue, Dunedin (NZ). DIAS, Subasinghe, Nisanke, George, Premalal, Jayantha [NZ/NZ]; 38 Scobie Road, Waverley, Dunedin (NZ). RODDICK-LANZILOTTA, Alisa, Dawn [NZ/NZ]; 11a Lyttelton Street, Lincoln, Christchurch (NZ). KELLY, Robert, James [GB/NZ]; 61 St Martin's Road, Christchurch (NZ).**

(74) Agent: **BALDWIN SHELSTON WATERS; PO Box 852, Wellington (NZ).**

(81) Designated States (*national*): AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SI, SK (utility model), SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- *with international search report*
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/103737 A1

(54) Title: **ORTHOPAEDIC MATERIALS DERIVED FROM KERATIN**

(57) Abstract: The invention provides a biocompatible material derived from keratin that is useful for many aspects of medical treatment of bone. The keratin material is preferably S-sulfonated and enriched in intermediate filament proteins of high molecular weight. The keratin material may be porous for use as a bone replacement and augmentation product but also provided is the use of dense keratin materials in bone treatment for use as an internal fixation appliance in the treatment of bone fractures and bone regeneration, and a method for preparing the keratin material for use in the preservation, restoration and development of form and function of bone.

BEST AVAILABLE COPY

ORTHOPAEDIC MATERIALS DERIVED FROM KERATINField of the Invention

This invention relates to the preparation of medical materials from keratins derived from animal sources such as wool, hair, horns, hooves, and scales. The keratin materials described are biocompatible, biointegratable, and biodegradable and the primary application of the materials is in orthopaedic surgery for replacement and augmentation of bone, and fixation and immobilization of bone fractures and bone segments.

10

Background to the Invention

Keratins are a class of structural proteins widely represented in biological structures, especially in epithelial tissues of higher vertebrates. Keratins may be divided into two major classes, the soft keratins (occurring in skin and a few other tissues) and the hard keratins (forming the material of nails, claws, hair, horn, feathers and scales).

The toughness and insolubility of hard keratins, which allow them to perform a fundamental structural role in many biological systems, are the desirable characteristics found in many of the industrial and consumer materials derived from synthetic polymers. In addition to possessing excellent physical properties, keratin, as a protein, is a polymer with a high degree of chemical functionality and consequently exhibits many properties that synthetic polymers cannot achieve. Keratin is therefore, well suited to the development of medical products with high-value, niche market applications. Medical materials which are absorbed (resorbed) by the body tissues after fulfilling their function are an example of an area of high value products in which the specific characteristics of keratin allow it to outperform both natural and synthetic competitive materials.

Yamauchi (K. Yamauchi, M. Maniwa and T. Mori, Journal of Biomaterial Science, Polymer edition, 3, 259, 1998) demonstrate that keratins can be processed into matrices that are considered biocompatible by virtue of their in vitro and in vivo properties. The processing methods used to make these materials require large concentrations of reducing agents, such as thiols, and processing conditions that are not suitable for commercial production of materials.

Kelly (WO03/19673) shows that keratins can be processed into complex shapes using commercially viable chemistries and processing conditions.

Blanchard (US5,358,935 and US2003/0035820A1) demonstrates that keratins can be
5 extracted from human hair using high concentrations of reductants, or harsh oxidants, and processed to produce materials useful in some soft tissue applications. However, extraction and reconstitution methods are harsh and cause degradation to the keratin, through irreversible oxidation of the distinctive keratin amino acid cystine to cysteic acid, or through exposure of the protein to high pH conditions that lead to peptide hydrolysis.
10 This results in many beneficial characteristics of the protein being lost, in particular the toughness necessary for hard tissue applications.

In order to produce keratin biomaterials suitable for orthopaedic applications, methods of processing are needed that maintain the keratin characteristics and provide materials with
15 good toughness properties. This invention describes such materials and their methods of production.

US 6,432,435 claims a tissue engineering scaffold having a keratin with hydrophilic groups, the keratin being bound with keratin-keratin disulfide bonds. The patent however
20 provides no disclosure as to how a sulfonated keratin can be incorporated into a hard tissue such as bone. The examples provided all relate to its use in soft tissue or porous structures.

The present inventors have found that keratin can be incorporated into hard tissue such
25 as bone and hence used in the treatment of bone injury.

Many tissues of the body including bone are continually renewed. New bone matrix (which will become mineralized) is laid down principally by specific cells called osteoblasts, and the different components of bone are removed by osteoclasts. An
30 implanted material which is removed and replaced with bone tissue by this biological process will have a greater advantage over those materials which break down by other mechanisms within the body e.g. chemical degradation. It is desirable that new bone is formed juxtaposed to the surface of the implanted material, thereby integrating this material into the tissue until it is completely resorbed and replaced.

Bone may be categorized into four microstructural components: cells, organic matrix, inorganic matrix, and soluble signalling factors. Osteoblasts are metabolically active secretory cells that express soluble signalling factors and osteoid, a product whose extracellular modification yields an organic insoluble substratum consisting mostly of type I collagen. Expression of these products by osteoblasts occurs during maintenance (e.g. remodelling), and repair of bone. Monocyte-macrophage precursors found in the bone marrow enter the circulation, and through asynchronous fusion produce a multinucleated cell up to 100 microns in diameter with an average of 10 to 12 nuclei, known as an osteoclast. Osteoclasts have a ruffled border and this constitutes the resorptive territory of the osteoclast where enzymatic breakdown of the bone surface occurs. The term 'remodelling' is used to describe the dynamic events associated with bone repair and homeostasis in the mature individual. The sum of the processes associated with homeostatic remodelling is known as activation-resorption-formation. Osteoblasts are activated by signalling factors and vacate an area of bone; osteoclasts become stimulated, home in to the osteoblast-vacant zone, attach, resorb, and, in response to an as yet unidentified signal, cease resorbing and abandon their attachment. Osteoclastic resorptive pits become repopulated by a contingent of osteoblasts that express osteoid, which calcifies, restoring bone. In humans, the activation-resorption-formation processes take between 3 and 6 months.

Following an insult to bone (e.g. fracture or surgical removal of a tumor) there is extensive bleeding and in 2 to 5 days the haemorrhage forms a large blood clot. Neovascularization begins to occur peripheral to this blood clot. There is also the standard inflammatory response occurring in the surrounding soft tissues leading to polymorphonuclear leucocytes, macrophages, and mononuclear cells accumulating in the periphery of the clot. By the end of the first week, most of the clot is organised by invasion of blood vessels and early fibrosis. The earliest bone (woven bone) is formed after 7 days. Since bone formation requires a good blood supply, the woven bone spicules begin to form at the periphery of the clot where vascularisation is greatest. Pluripotential mesenchymal cells from the surrounding soft tissues and from within the bone marrow give rise to osteoblasts that synthesize the woven bone. Frequently cartilage is also formed and eventually is replaced by endochondral ossification. The granulation tissue containing bone-cartilage is termed a callus (Inflammatory phase).

After the first week, the next stage begins and extends for several months, depending upon the degree of movement and fixation. By this stage, the acute inflammatory cells have dissipated and the reparative process involving the differentiation of pluripotential cells into fibroblasts and osteoblasts commences. Repair proceeds from the periphery 5 towards the centre and accomplishes two objectives: one, it organises and resorbs the blood clot; and two, more importantly, it furnishes neovascularisation for the construction of the callus, which eventually bridges the bone-deficient site. The events leading to the repair are as follows. Large numbers of osteoclasts from the surrounding bone move into the healing site. New blood vessels accompany these cells supplying nutrients and 10 providing more pluripotential cells for cell renewal. The site is remodelled by osteoclasts (Reparative phase).

In several weeks the callus has sealed the bone ends and remodelling begins, in which the bone is reorganised so that the original cortex is restored (Remodelling phase).

15

Object of the invention

It is an object of the invention to provide a material which contains keratin and which is useful in assisting bone formation and/or which provides the public with a useful choice.

20

Summary of the Invention

The invention provides a keratin-containing material for use in preservation, restoration and development of form and function of bone in the skeletal system.

25

The invention also provides a porous keratin material for use in the replacement and augmentation of bone.

The invention also provides a dense keratin material for use in bone fixation and 30 immobilization.

The keratin is preferably S-sulfonated and more preferably is enriched in intermediate filament protein.

The dense keratin material may be prepared by compression of solid keratin powder or by compression of keratin films.

The material may contain up to 60% calcium salts.

5

The material may be prepared by compression and freeze-drying of solid keratin.

Alternatively a solution of keratin may be freeze dried.

10 The invention also provides the use of a keratin material in the preservation, restoration and development of form and function of bone.

The invention also provides a method of forming a porous S-sulfonated keratin enriched material comprising:

- 15 a) compressing keratin in the presence of a soluble porogen;
b) removing the porogen and strengthening the material;
c) washing the protein material; and
d) freeze drying the material.

20 The invention also provides a method of forming keratin material into an orthopaedic product.

25 The invention also provides a biocompatible material in the form of a porous keratin that is enriched in intermediate filament protein for use in bone replacement / augmentation therapy.

30 The material may be prepared by compression of solid keratin powder, in the presence of a porogen and compression may be followed by freeze drying. The porogen may be selected from sodium chloride or another biocompatible salt, or glycerol or another biocompatible solvent. The amount and nature of porogen may be controlled to select the pore sizes and allow the infiltration of osteoprogenitor cells to facilitate the colonization of keratin material when implanted. Hydroxyapatite may also be added to the keratin.

35 The invention also provides a method of forming a dense material of S-sulfonated keratin material into an orthopaedic product comprising:

- a) compressing keratin in the presence of heat and water;
- b) strengthening the material;
- c) washing the material to remove residual chemicals; and
- d) drying the material.

5

The invention also provides a method for forming a dense material of S-sulfonated keratin into an orthopaedic product comprising:

- a) strengthening the keratin-containing starting material;
- b) washing the material to remove residual chemicals;
- c) drying the material; and
- d) compressing keratin in the presence of heat and water.

10

The invention also provides orthopaedic medical materials such as plates, pins and screws manufactured from biocompatible keratin material for treatment of fractures by internal fixation as well as fixation and immobilisation of bone segments.

15

The invention also provides a method of reforming S-sulfonated keratin enriched in intermediate filament protein into a tough, dense biocompatible material for use as an internal fixation appliance in the treatment of bone fractures.

20

Moisture and chemicals may be added. The controlled use of reducing agents may be used to remove the sulfonate group from the S-sulfonate keratin and reform disulfides originally present.

25

The keratin used in the formation of bone can be made by the method according to WO 03/011894 which provides a process for the preparation of keratin derivatives of high molecular weight, whereby the process includes a first stage digestion step of sulfonating a keratin source by oxidative sulfitolysis followed by a second stage repetitive aqueous extraction involving separation of soluble and insoluble keratin and subsequent re-extraction of the insoluble keratin to thereby produce a highly S-sulfonated keratin derivative.

30

WO 03/018673 provides a foam fibre adhesive material or film derived from S-sulfonated keratin protein. The method involves solvent casting a solution of S-sulfonated keratin protein.

35

Also provided is a method for making a film, fibre, foam or adhesive material derived from highly S-sulfonated keratin intermediate filament proteins.

- 5 The present invention provides a method of making keratin material suitable for use in bone structures and also provides keratin material for use in bone structures based on the methods of WO 03/018673 and WO 03/011894.

A degradable keratin appliance will provide enough flexibility to stimulate new bone
10 growth (unlike some rigid permanent materials currently used) by gradually transferring functional loads to the healing bone. Furthermore subsequent surgery is not needed to remove the keratin-derived fixation appliances or devices.

The method of reforming the S-sulfonated keratin enriched in intermediate filament protein
15 into a tough, dense material for use as an internal fixation appliance in the treatment of bone fractures, may involve compressing the biocompatible protein in the presence of moisture, chemicals, and in some cases heat, to form a desired shape. The formation of crosslinks within the material to ensure strength and toughness under biological conditions occurs during compression, or subsequently through chemical treatment.

20 The method may also involve the controlled use of reducing agents to remove the sulfonate group from the S-sulfonated keratin and reform the disulfides originally present in the native keratin. This serves the dual purpose of firstly, providing strength and toughness under biological conditions by effectively polymerizing the keratin protein
25 through an extended network of disulfide crosslinks; and secondly, controlling the rate and extent of biodegradation that occurs by impeding enzymatic digestion of the material. By controlling the rate of biodegradation, the invention allows the keratin products to be used in applications where a range of healing times are desirable.

30 The reconstituted keratin described in this patent specification is completely biocompatible and does not elicit any significant foreign body type immune reaction. The keratin is degraded by the normal processes occurring in bone described above and is replaced with normal bone. Therefore in bone replacement and augmentation, this keratin has similar properties to autologous bone. In the case of treatment of fractures, appliances
35 made of this keratin will have the necessary physical properties to carry out the fixation

and immobilization of the bone segments, and once this function is fulfilled the keratin will gradually resorb and eventually disappear from the tissues. During the initial period of resorption, the gradually weakening keratin appliance will stimulate new bone formation by subjecting the bone to increasing functional loads. This will prevent a stress shielding effect which is found with metal appliances. Furthermore the degradation and resorption of keratin is a great advantage compared to metal appliances which are either permanently retained in the body or require a second surgical procedure to remove them.

The invention may be applied to any bone formation function. Form and function of bones can be disrupted by:

- developmental anomalies where bone is under developed, or abnormally developed
- trauma causing either fractures, fracture dislocations, with or without loss of bone
- surgical interventions such as, excision of malignant or benign tumours/tumour-like conditions, or in degeneration conditions of bone
- bone necrosis and destruction caused by conditions such as infections

The treatment modalities to these conditions attempt to preserve, restore, or develop the normal form and function of the affected bones of the skeletal system.

The term "intermediate filament proteins" describes keratin proteins from the intermediate filament protein class. Keratin intermediate filaments are derived from hard alpha keratins and generally recognized in the art as comprising of two families of low sulphur polypeptides containing both helical and non-helical segments, as discussed in Marshall et al, Structure and Biochemistry of Mammalian Hard Keratin, Electron Microscopy Review, Vol 4, pp 47-83, 1991.

"Enriched in intermediate filament protein" means that there is more intermediate filament protein in the product than in the corresponding amount of native keratin source.

30

Description of preferred embodiments of the invention

The invention will now be described, by way of example only with reference to the following preferred embodiments.

35

The features of this invention specifically cite some methods and applications based on hard α -keratins from wool. However, the principle can equally well apply to alternative α -keratins, or any source of keratin which is able to yield proteins of the intermediate filament (IF) type.

5 Similar preparative methods have been applied by the applicants to other keratin sources such as feathers, to produce materials equally well suited for some of the applications described below. The features of this invention are extended to cover limited areas of the utilisation of such keratins as well, in applications which are not dependent on the
10 presence of proteins of the α -type (IF proteins). This includes applications where preparations based on β or feather keratin may be combined with IF proteins.

Wool represents a convenient source of hard α -keratins, although any other animal fibre, or horns, or hooves, would serve equally well as a source of the desired proteins. Wool is
15 composed of approximately 95% keratin, which can be broadly divided into three protein classes. The intermediate filament proteins are typically of high molecular weight (45-60kD), with a partly fibrillar tertiary structure and a cysteine content of the order of 6%. They account for approximately 58% of the wool fibre by mass although only part of this mass is actually helix-forming in structure. The high- and ultra-high-sulphur proteins,
20 approximately 26% of the wool fibre, are globular in structure, have a molecular weight range of 10-40kD and can contain cysteine levels up to 30mol%. The high-glycine-tyrosine proteins are a minor class comprising 6% of the wool fibre, have molecular weights of the order of 10kD and are characterised by their high content of glycine and tyrosine amino acid residues.

25 Proteins from the different classes of wool keratins possess characteristics that will give them unique advantages in specific applications.

This invention pertains largely to the use of intermediate filament proteins, and the use of
30 them to produce materials for use in orthopaedic surgery

Nonetheless the other non-fibrillar proteins have applications in their own right in more restricted fields.

Highly S-sulfonated keratin, derived from an animal source such as those described, when purified to isolate the intermediate filament protein component, is a biocompatible/biodegradable material ideal for use in a range of products for orthopaedic use. The keratin may be prepared by methods such as those outlined in WO 03/011894.

5 The S-sulfonated keratin proteins themselves may be made according to methods in WO 03/018673. These methods result in the cystine present in the original keratin source being modified to become S-sulfocysteine. This group is highly polar and can lead to the derivatised keratin becoming soluble under aqueous conditions at pH >4. In addition the S-sulfonated material is responsive to moisture and suitable for processing into matrices
10 useful in orthopaedic care. In some cases it is useful to retain the S-sulfonate functionality, whereas for other matrices it is useful to chemically treat the protein as part of a reconstitution process to remove the S-sulfonate functionality and restore the disulfide bonds originally present within the keratin. As well as being highly polar and therefore responsive to moisture, the presence of S-sulfo derivatisation renders reconstituted
15 keratin materials susceptible to enzymatic degradation by the proteolytic enzymes present in vivo. Conversion of the S-sulfo groups to cystine renders the prepared keratin materials insoluble and also imparts a degree of resistance to proteolytic enzymes. The cystine containing materials are therefore degraded at a slower rate within the body, a performance characteristic that is important in some orthopaedic applications.

20 The conversion of S-sulfocysteine to cystine can occur through the application of reducing agents, typically thiol containing solutions such as ammonium thioglycollate, or through methods such as those described in WO 03/018673.

25 As a proteinaceous material, keratin is well suited as a material used for the creation of orthopaedic care materials which incorporate osteogenic agents, such as the well documented osteogen hydroxyapatite. Reconstituted keratin materials can be readily prepared containing hydroxyapatite at levels from 0-60%, depending on the method of reconstitution. The beneficial healing effects of hydroxyapatite inclusion in biomaterials is
30 well documented, and also demonstrated as part of this invention.

In one embodiment of the invention the S-sulfonated keratin is reconstituted into a porous material for use as a bone replacement or augmentation product. This is achieved by compressing the keratin protein in the presence of water and a soluble porogen, such as
35 sodium chloride. Generally, S-sulfonated keratin intermediate ground to a particle size to

- aid powder compression, preferable of 125-200 micron, is mixed with water in the ratio from 1:0.1 to 1:10, preferably 1:1, and also sodium chloride in the ratio 1:0.01 to 1:10. Hydroxyapatite is also incorporated into this mixture at a level of 0-60% by mass, preferably 0-10%. This mixture is packed into a die and pressed to a pressure in the range 10,000 to 50,000 kPa for a time in the range 1-30 minutes. On removal from the die a cylinder is cut from the pellet and the cylinder is soaked in a chemical treatment solution, in order to wash out the porogen and leave a porous material, while also removing the sulfonate functionality from the keratin and restoring disulfide crosslinks to the protein. This chemical treatment may take one of two forms. Firstly, a solution of ammonium thioglycollate preferably of concentration 0.25M, containing sodium phosphate, preferably of concentration 0.1M buffered to pH 7.0 can be used to treat the cylinder for a period of from 2-48 hours, preferably 18 hours. Residual chemicals are removed by subsequent washing of the pellet in water. After washing the cylinder is then freeze dried. Alternatively, the cylinder can be subjected to chemical treatment in a solution containing thioglycollic acid, preferably 0.1M for 2-48 hours, preferably 18 hours. Following a brief wash in water, residual chemicals are removed by washing the cylinder in a buffered solution 48 – 96 hours. The solution is refreshed every 24 hours. Following a further wash in water the cylinder is freeze dried.
- The pore size in the material can be controlled by varying the quantity of sodium chloride used in the preparation. For example, typical pore size for a preparation using 0.06g of sodium chloride per gram of protein is 50-150 microns, whereas 0.14g of sodium chloride per gram of protein results in pores of up to 320 microns.
- The biological properties of the porous keratin material prepared using the thioglycollic acid method are demonstrated both *in vitro* and *in vivo*. *In vitro*, the material is not cytotoxic and supports the growth of human and sheep fibroblasts. Direct contact of the porous material with cells, as described in ISO 10993-5, using sheep fibroblasts produced the following effects. Wells of polystyrene cell culture plates containing either the porous material or no material as a control were initially seeded with ~10,000 cells (0 hours). During the first 24h post-seeding, the cultures experienced a lag time as evidenced by a decline in cell numbers. This phenomenon has been recognised in all assays performed and the drop is observed in control wells in addition to those containing the test materials. Experimentation has shown that this lag time lasts for less than 12h and that the exponential phase of growth begins at this point. Population doublings occur

approximately every 24h-48h with subconfluence (approximately 80% confluency) marking the end of logarithmic growth. This corresponds to the end of the experimental time course (5 days or 120h). Extended time-course experiments have indicated a plateau in cell growth shortly after this with full confluence of the culture. Contact inhibition and depletion of nutrients play a key role in limiting the growth rate at this point and the monolayer culture exhibits signs of cell death (i.e. loss of membrane integrity, reduction in cell numbers, vacuolisation of individual cells). During the assay, cells were witnessed to attach to the upper surface of the disks. By light microscopy, the morphological appearance of these cells was deemed similar on all substrates compared to the no-material control. Similar assays with human fibroblasts produced very similar results, with typical fibroblast growth curves occurring in the presence of the porous material and approximately 80% confluency reached after 120 hours in culture. The control wells had reached 100% confluency at this time.

The in vivo study involved the keratin material treated by the above method, and also a composite of this material containing 6% hydroxyapatite, and manufactured as rods 3 mm diameter, 3 mm length and sterilized by gamma radiation (2.8 Mrads) being implanted into the midshaft (cortical bone) and proximal and distal ends (cortico-cancellous bone) of the long bones of the hind limbs of adult sheep. The tissue responses to the implanted material were studied by histological examination of biopsy samples at 10 days, 3, 6, 8, 12 and 24 weeks. The bone tissue response to the material prepared using thioglycollic acid showed minimal foreign body type immune reaction to the presence of the implant and there was only a thin layer of granulation tissue (leading to fibrosis) formed between the implant and the surrounding bone. Within 3 to 6 weeks the implant material was colonized by osteoid tissue leading to the laying down of woven bone in the spaces created by resorption of the keratin implant material, and the new bone was joined to the surrounding bone at around 6 to 8 weeks. From 6 weeks onwards remodelling of woven bone into corticocancellous bone occurred. The continuation of this process led to complete integration of the implant material which was replaced by mature bone, and the bone defect was completely healed.

Furthermore the physical properties of this porous material were investigated by manufacturing it in the form of plates 12 mm length, 4 mm width, 3 mm depth and implanted subcutaneously in the sheep. The data obtained by testing the plates on an Instron machine showed that the tensile properties weakened by approximately 10% over

a period of 3 to 6 weeks. This was consistent with a loss in dry weight of about 10% at this time. These findings support the ability of this material to stimulate new bone formation and prevent stress shielding, and gradual resorption of this material.

- 5 Porous keratin material are also prepared by freeze drying an aqueous solution of S-sulfonated keratin, using methods such as those described in WO 03/018673, and these materials are suitable for use in bone graft applications. A porous keratin material containing a high proportion of hydroxyapatite is prepared from an aqueous solution of S-sulfonated keratin, preferably 5%, by suspending insoluble hydroxyapatite in the keratin
10 solution at a ratio from 1:0.1 to 1:2 keratin to hydroxyapatite mass, preferably 1:1. Upon freezing and subsequent freeze drying an intimate mixture of keratin and hydroxyapatite is created, in a porous sponge material suitable for use in bone graft products.

In another embodiment of the invention the S-sulfonated keratin is reconstituted into a
15 tough, dense material for use as a bone fixation product. This is achieved by compressing the keratin protein. Generally, S-sulfonated keratin ground to a particle size to aid powder compression, preferable of 125-200 micron, is mixed with water in the ratio from 1:0.1 to 1:10, preferably 1:1. Hydroxyapatite is also incorporated into this mixture at a level of 0-60% by mass, preferably 0-10%. This mixture is packed into a die and
20 pressed to a pressure in the range 10,000 to 50,000 kPa for a time in the range 1-30 minutes. On removal from the die a cylinder is cut from the pellet and the cylinder is soaked in a chemical treatment solution, in order to wash out the porogen and leave a porous material, while also removing the sulfonate functionality from the keratin and restoring disulfide crosslinks to the protein. This chemical treatment may take one of two
25 forms. Firstly, a solution of ammonium thioglycollate preferably of concentration 0.25M, containing sodium phosphate, preferably of concentration 0.1M buffered to pH 7.0 can be used to treat the cylinder for a period of from 2-48 hours, preferably 18 hours. Residual chemicals are removed by subsequent washing of the pellet in water. After washing the cylinder is then freeze dried. Alternatively, the cylinder can be subjected to chemical
30 treatment in a solution containing thioglycollic acid, preferably 0.1M for 2-48 hours, preferably 18 hours. Following a brief wash in water, residual chemicals are removed by washing the cylinder in a buffered solution 48 – 96 hours. The solution is refreshed every 24 hours. Following a further wash in water the cylinder is freeze dried.

The biological properties of the dense keratin material were demonstrated *in vitro* and the biophysical properties were examined *in vivo*. *In vitro*, the material is not cytotoxic and supports the growth of human and sheep fibroblasts. In a similar manner to that outlined above for the porous matrices, direct contact of the porous material with cells, as described in ISO 10993-5, using sheep fibroblasts produced the following effects. Wells of polystyrene cell culture plates containing either the non-porous material or no material as a control were initially seeded with ~10,000 cells (0 hours). During the first 24h post-seeding, the cultures experienced a lag time as evidenced by a decline in cell numbers. This phenomenon has been recognised in all assays performed and the drop is observed in control wells in addition to those containing the test materials. Experimentation has shown that this lag time lasts for less than 12h and that the exponential phase of growth begins at this point. Population doublings occur approximately every 24h-48h with subconfluence (approximately 80% confluency) marking the end of logarithmic growth. This corresponds to the end of the experimental time course (5 days or 120h). Extended time-course experiments have indicated a plateau in cell growth shortly after this with full confluence of the culture. Contact inhibition and depletion of nutrients play a key role in limiting the growth rate at this point and the monolayer culture exhibits signs of cell death (i.e. loss of membrane integrity, reduction in cell numbers, vacuolisation of individual cells). During the assay, cells were witnessed to attach to the upper surface of the disks. By light microscopy, the morphological appearance of these cells was deemed similar on all substrates compared to the no-material control. Similar assays with human fibroblasts produced very similar results, with typical fibroblast growth curves occurring in the presence of the porous material and approximately 80% confluency reached after 120 hours in culture. The control wells had reached 100% confluency at this time.

The biophysical properties (modulus of elasticity, modulus of rupture, tensile strength) were tested by implanting the material treated with thioglycollic acid and manufactured as plates 12 mm length, 4 mm width, 3 mm depth subcutaneously in adult rats. The plates were removed from the rats at 1, 3, 6 and 12 weeks and the physical strengths evaluated. The modulus of elasticity showed a decrease of 40 to 70% over a period of 3 to 6 weeks. The loss in dry weight at these two times was 5 to 10% which was in agreement with our study in the sheep with plates of the porous material.

Another aspect of the invention is a tough, dense keratin material for use in the fixation of bone, constructed from the heated compression of multiple layers of keratin film. Keratin

films are readily prepared from S-sulfonated keratin using methods such as those described in WO 03/018673. Keratin films can also be constructed using a solution of S-sulfonated keratin which contains a suspension of hydroxyapatite. Using this method hydroxyapatite is incorporated into keratin films at a level of 0-50% on mass of keratin.

5 The films formed can be stacked into layers and compressed to a pressure in the range 10,000 to 100,000 kPa at a temperature in the range 50 to 200 °C for a time in the range 1 to 60 minutes. Following compression water is added to the compressed mixture and further compression occurs, under identical conditions. The resulting material contains an intimate mixture of keratin and hydroxyapatite in the form of a dense, tough block.

10 Chemical treatment is then used to convert the S-sulfonate groups back to cystine. Thioglycolic acid or ammonium thioglycollate is used, under treatment and washing conditions identical to those described above. Once dry, the resulting material can be machined to a shape useful as a device in orthopaedic surgery, such as a screw or a pin.

15 In an alternative approach, chemical treatment is applied to keratin films containing hydroxyapatite at a level of 0-60% prior to stacking them into layers. Chemical treatment is achieved by soaking the films in a solution of ammonium thioglycollate of similar composition to that described above, for a period of 20 – 60 minutes. Multiple washing of the films in water removes residual chemical and on drying the resulting materials contain
20 keratin with the S-sulfo group having been converted back to cystine. The films are then layered and compressed in a manner identical to that described above and the resulting material contains an intimate mixture of keratin and hydroxyapatite in the form of a dense, tough block. No further chemical treatment is needed, and the material can be machined to a shape useful as a device in orthopaedic surgery, such as a screw or a pin.

25 An aspect of the invention is the use of reducing agents, such as ammonium thioglycollate or thioglycolic acid described above, in order to remove the sulfonate functionality from the protein and restore the disulfide bonding originally present in the native keratin. In the sulfonate form the keratin is soluble above pH 4 and rapidly resorbed *in vivo*. In order to
30 sustain the material for a longer time within the body, and control the rate of degradation and resorption of the material, reductive agents that remove the sulfonate function and crosslink the protein can be employed. The extent to which reductive agents are used, the time of exposure and concentration of reagents, affects the ratio of sulfonate groups to disulfide bonds present within the material. This in turn affects the strength and rate of
35 degradation *in vivo*. Other crosslinking agents, such as those employed to modify the

properties of other biological biomaterials, for example glutaraldehyde and ethyldimethylaminopropylcarbodiimide hydrochloride (EDC) which are used to modify the properties of collagen biomaterials, can also be used to modify the properties of the keratin biomaterials.

5

Examples

Examples

Example 1a: Porous keratin material.

10 0.4g of S-sulfonated keratin intermediate filament protein powder, ground to a particle size of 125-300 micron, is mixed with 0.5ml of water and 0.024g of sodium chloride and stand for 5 minutes. This mixture is packed into a 12mm diameter die and pressed to a pressure of 15,000kPa for 2 minutes. On removal from the die a 3mm diameter cylinder is cut from the pellet and the cylinder is soaked in a chemical treatment solution, in order to wash out
15 the porogen and leave a porous material, while also removing the sulfonate functionality from the keratin and restoring disulfide crosslinks to the protein. This chemical treatment may take one of two forms. Firstly, a solution of 0.25M ammonium thioglycollate containing 0.1M sodium phosphate buffered to pH 7.0 can be used to treat the cylinder for a period of 18 hours. Residual chemicals are removed by subsequent washing of the
20 pellet in water successively for 10, 40 and 10 minute periods. After washing the cylinder is then freeze dried. Alternatively, the cylinder can be subjected to chemical treatment in a solution containing 0.1M thioglycollic acid for 18 hours. Following a brief wash in water, residual chemicals are removed by washing the cylinder in a solution of 0.1M TRIS 11.25 mM calcium chloride for 72 hours. The solution is refreshed every 24 hours. Following a
25 further wash in water the cylinder is freeze dried.

Example 1b: Porous keratin material containing hydroxyapatite.

Production of this material is identical to that described in example 1a, with the addition of 0.034g hydroxyapatite at the same point as the inclusion of sodium chloride, to give the
30 final product a level of 6% hydroxyapatite by mass.

Example 1c: Porous keratin material containing hydroxyapatite.

In order to prepare a porous keratin material, a 5% keratin solution was prepared by suspending 0.5g S-sulfonated wool keratin protein in water, followed by the gradual
35 addition of 0.5ml of 1M sodium hydroxide to the vigorously stirred solution over the

approximately 2 hours. The pH of the solution was carefully monitored and observed to elevate to ~pH10 upon immediate addition of base, and gradually fall as the base was absorbed by dissolution of the protein. A final pH of 9.5 was obtained. The protein solution was centrifuged at 34,000g to remove any insoluble material and 0.5g of hydroxyapatite
5 was thoroughly mixed into the solution. The mixture was frozen and freeze dried to produce a porous material. The material was immersed in an ammonium thioglycollate solution identical to that used in example 1a for 30 minutes, and subsequently washed in three batches of water for 20 minutes and freeze dried.

10 **Example 2a: Dense keratin material**

0.5g of S-sulfonated keratin protein powder, ground to a particle size of 125-200 micron, is mixed with 0.5ml of water and allowed to stand for 5 minutes. This mixture is packed into a 12 mm diameter die and pressed to a pressure of 15,000 kPa for 2 minutes. On removal from the die a desirable shape is cut from the pellet, such as a 12mm by 4 mm block. The
15 block is soaked in a chemical treatment solution, in order to remove the sulfonate functionality from the keratin and restore disulfide crosslinks to the protein. This chemical treatment may take one of two forms. Firstly, a solution of 0.25M ammonium thioglycollate containing 0.1M sodium phosphate buffered to pH 7.0 can be used to treat the block for a period of 18 hours. Residual chemicals are removed by subsequent washing of the block
20 in water successively for 10, 40 and 10 minute periods. After washing the block is then allowed to dry in air at room temperature. Alternatively, the block can be subjected to chemical treatment in a solution containing 0.1M thioglycolic acid for 18 hours. Following a brief wash in water, residual chemicals are removed by washing the block in a solution of 0.1M TRIS 11.25mM calcium chloride for 48 hours. The solution is refreshed every 24
25 hours. Following a further wash in water the block is dried in air at ambient temperature.

Example 2b: Dense keratin material containing hydroxyapatite

Production of this material is identical to that described in example 2a, with the addition of 0.032g hydroxyapatite to the keratin powder at the start of the process, to give the final
30 product a level of 6% hydroxyapatite by mass.

Example 2c: dense keratin material constructed from keratin films

Films of S-sulfonated keratin, of thickness 0.3mm prepared by methods such as those outlined in WO 03/018673 were cut to 4x50mm. A number of films of total mass 1g
35 (equivalent to 50 films) were stacked in layers in a heated steel block of internal

dimensions 5x50mm and pressed to a pressure of 50,000kPa for 5 minutes, maintained at a temperature of 80-100°C through heating of the steel block. The keratin block formed was immersed in the ammonium thioglycollate solution described in example 1a for 18 hours, and subsequently washed in water for 10,40,10 minutes. Air dry

5

In a variation, the films used contained 50% hydroxyapatite and were prepared as follows: 1.0g S-sulfonated wool keratin protein was suspended in water 0.2g of Glycerol and 1.0ml of 1M sodium hydroxide was gradually added to the vigorously stirred solution over the approximately 2 hours. The pH of the solution was carefully monitored and observed to 10 elevate to ~pH10 upon immediate addition of base, and gradually fall as the base was absorbed by dissolution of the protein. A final pH of 9.5 was obtained. The protein solution was centrifuged at 27,000g to remove any insoluble material. 1.0g of hydroxyapatite was made into a paste with a small amount of 99% ethanol(~1ml)and then thoroughly mixed in with the 5% solution. The mixture was cast on a 100mm square petri dish and allowed to 15 dry under ambient conditions.

Example 2d: Dense keratin material constructed from pretreated keratin films

Films of S-sulfonated keratin, of thickness 0.3mm prepared by methods such as those outlined in WO 03/018673 were treated in the solution of ammonium thioglycollate 20 described in example 1a for 30 minutes. Following treatment the films were washed in water and dried to leave films containing disulfide rather than S-sulfonate functionality. These films were cut to 4x50mm. A number of films of total mass 1g (equivalent to 70 25 films) were stacked in layers in a heated steel block of internal dimensions 5x50mm and pressed to a pressure of 50,000kPa for 5 minutes, maintained at a temperature of 80-100°C through heating of the steel block. Remove the keratin block and briefly soaked in water and press into the heat block and compress for 20,000kpa for 5 minutes. Heat the block to 80-100°C under pressure. Press to 30,000kPa for 30 minutes.

In a variation, the films used contained 50% hydroxyapatite and were prepared as 30 described for example 2b.Dense keratin material with hydroxyapatite were prepared as same as 2c

Where in the foregoing description, reference has been made to specific components or integers of the invention having known equivalent, then such equivalents are herein

incorporated as if individually set forth.

Industrial Applicability

The invention will be useful in the medical field, especially in the area of bone injury.

- 5 Keratin materials according to the invention are biocompatible and can be used as bone replacement and augmentation products.

The product can be used for example to replace bone lost due to clinical conditions such as trauma or tumors and will promote healing by acting as a scaffold for the laying down
10 of new bone. The porous keratin scaffold is resorbed and replaced by new woven bone which will subsequently be remodelled into cancellous and cortical bone.

CLAIMS

1. A keratin-containing material for use in the preservation, restoration and
5 development of form and function of bone.

2. A porous keratin material for use in the replacement and augmentation of bone.

3. A dense keratin material for use in bone fixation and immobilization.

10 4. A material according to any of claims 1, 2 or 3 wherein the keratin is S-sulfonated.

15 5. A material according to any one of claims 1-4 wherein the keratin is enriched in intermediate filament protein.

6. The keratin material of claim 5 which is prepared by compression of solid keratin powder.

20 7. The dense material of claim 3 which is prepared by compression of keratin film.

8. The material of any one of claims 1-7 that contains up to 60% calcium salts.

25 9. The material of any one of claims 6 or 7 wherein compression is followed by freeze-drying of solid keratin.

10. A use of a dense keratin material in the manufacture of a medical support or scaffold in the preservation, restoration and development of form and function of bone.

30 11. The use according to claim 10 wherein the keratin material is S-sulfonated.

12. The use according to claim 10 or 11 wherein the keratin is enriched in intermediate filament protein.

35 13. A method of forming a dense material of S-sulfonated keratin material into an orthopaedic product comprising:

- a) compressing keratin in the presence of heat and water;
- b) strengthening the material;
- c) washing the material to remove residual chemicals; and
- d) drying the material.

5

14. A method for forming a dense material of S-sulfonated keratin into an orthopaedic product comprising:

- a) strengthening the keratin-containing starting material;
- b) washing the material to remove residual chemicals;
- c) drying the material; and
- d) compressing keratin in the presence of heat and water.

10

15. A method of forming a porous S-sulfonated enriched keratin material comprising:

- a) compressing keratin in the presence of a soluble porogen;
- b) removing the porogen and strengthening the material;
- c) washing the protein material; and
- d) freeze drying the material.

15

16. A method according to claim 15 wherein the porogen is selected from sodium chloride or another biocompatible salt, or glycerol or another biocompatible solvent.

20

17. A method according to any one of claims 15-16 wherein the amount and nature of porogen is controlled to select the pore sizes and allow the infiltration of osteoprogenitor cells to facilitate the colonization of keratin material when implanted.

25

18. A method according to any of claims 13-17 further including the addition of hydroxyapatite to the keratin starting material.

30

19. A method according to any one of claims 13-18 wherein the keratin is enriched in intermediate filament protein.

20. A keratin material prepared by the method of any one of claims 13-19.

35

21. A biocompatible material in the form of a porous keratin that is enriched in intermediate filament protein for use in bone replacement / augmentation therapy.

22. A biocompatible material according to claim 21 wherein the keratin is S-sulfonated.

23. A biocompatible material according to claim 21 or 22 which contains up to 60% calcium salts.

24. A biocompatible material according to any one of claims 21-23 wherein the material is prepared by compression of solid keratin powder.

25. A biocompatible material according to claim 24 wherein compression is followed by freeze-drying.

26. A biocompatible material according to any one of claims 21-25 wherein the material is prepared from a solution of keratin.

27. A biocompatible material according to claim 26 wherein the solution of keratin is freeze-dried.

28. An orthopaedic medical material manufactured from biocompatible keratin material for treatment of fractures by internal fixation as well as fixation and immobilisation of bone segments.

29. An orthopaedic medical material according to claim 28 which is manufactured from S-sulfonated keratin material.

30. An orthopaedic medical material according to claim 28 or 29 wherein the keratin material is enriched in intermediate filament protein.

31. An orthopaedic medical material according to any one of claims 28-30 prepared by compression of solid keratin powder.

32. An orthopaedic medical material according to any one of claims 28-30 prepared by compression of keratin film.

33. An orthopaedic medical material according to any one of claims 28-30 prepared from a solution of keratin.

5 34. An orthopaedic medical material according to any one of claims 28-30 that contains up to 60% calcium salts.

10 35. An orthopaedic medical material according to any one of claims 31-33 wherein the keratin is freeze dried after compression.

36. An orthopaedic material according to any one of claims 28-35 made according to the method of any one of claims 13-19.

15 37. A method of reforming S-sulfonated keratin enriched in intermediate filament protein into a tough, dense biocompatible material for use as an internal fixation appliance in the treatment of bone fractures.

20 38. A method according to claim 36 wherein the keratin is enriched in intermediate filament protein.

25 39. A method according to claim 37 that includes compressing the biocompatible protein in the presence of moisture and chemicals.

40. A method according to claim 39 wherein heat is also used to form a desired shape.

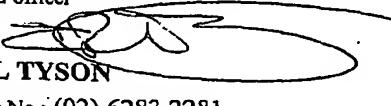
25 41. A method according to any one of claims 37-40 that also involves the controlled use of reducing agents to remove the sulfonate group from the S-sulfonated keratin and reform the disulfides originally present in the native keratin.

30 42. A biocompatible keratin enriched material when produced according to any one of claims 37-41.

35 43. An orthopaedic material according to claim 28 wherein the material is a plate, pin or screw.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ03/00116

A. CLASSIFICATION OF SUBJECT MATTER																						
Int. Cl. ⁷ : A61L 27/22, 27/46, 27/56, C08H1/06, C08L 89/04, C08K 3/32																						
According to International Patent Classification (IPC) or to both national classification and IPC																						
B. FIELDS SEARCHED																						
Minimum documentation searched (classification system followed by classification symbols)																						
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched																						
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WPAT, JAPIO: keratin, bone#; Medline, CA: keratin, bone#, cellular, porous,																						
C. DOCUMENTS CONSIDERED TO BE RELEVANT																						
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																				
X	WO 99/26570 A (KERAPLAST TECHNOLOGIES LTD), 03 June 1999 - see whole document	1-43																				
X	WO 99/19005 A (ED GEISTLICH SÖHNE AG FÜR CHEMISCHE INDUSTRIE), 22 April 1999 - see whole document	1-43																				
X	WO 2000/41739 A (UNIVERSITY OF UTAH RESEARCH FOUNDATION), 20 July 2000 - see whole document	1-43																				
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex																						
<p>* Special categories of cited documents:</p> <table> <tr> <td>"A"</td> <td>document defining the general state of the art which is not considered to be of particular relevance</td> <td>"T"</td> <td>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"E"</td> <td>earlier application or patent but published on or after the international filing date</td> <td>"X"</td> <td>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"L"</td> <td>document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"Y"</td> <td>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"O"</td> <td>document referring to an oral disclosure, use, exhibition or other means</td> <td>"&"</td> <td>document member of the same patent family</td> </tr> <tr> <td>"P"</td> <td>document published prior to the international filing date but later than the priority date claimed</td> <td></td> <td></td> </tr> </table>			"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family	"P"	document published prior to the international filing date but later than the priority date claimed		
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention																			
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone																			
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art																			
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family																			
"P"	document published prior to the international filing date but later than the priority date claimed																					
Date of the actual completion of the international search 3 October 2003	Date of mailing of the international search report 22 OCT 2003																					
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaaustralia.gov.au Facsimile No. (02) 6285 3929	Authorised officer  ISOBEL TYSON Telephone No : (02) 6283 2281																					

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ03/00116

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2002013408 A (RHEE, W.M.), 31 January 2002 - see whole document	1-43
X	US 2001018614 A (BIANCHI, J.R.), 30 August 2001 - see whole document	1-43
X	US 5972385 (LIU, L. et al.), 26 October 1999 - see whole document	1-43
X	US 2002183858 A (CONTILIANO, J.H.), 5 December 2002 - see whole document	1-43
X	US 2003039676 A (BOYCE, T.M.), 27 February 2003 - see whole document	1-43
X	JP 63229058 A (OSHIMA, Y.), 22 September 1988 - see abstract	1-43
X	FR 2687577 A (ICP FRANCE), 27 August 1993 - see whole document	1-43

International application No.

PCT/NZ03/00116

INTERNATIONAL SEARCH REPORT

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report			Patent Family Member			
WO 9926570	EP	1033953	EP	1039873	US	5932552
	US	5948432	US	6110487	US	6124265
	US	6159495	US	6159496	US	6165496
	US	6274163	US	6432435	US	2001009675
	US	2003035820	WO	9926595	WO	9951175
WO 9919005	CA	2305740	EP	1023091	PL	339835
	US	2002177903				
WO 0041739	AU	24143/00				
US 2002013408	AU	13344/97	AU	70843/00	EP	0876165
	EP	1218437	US	5874500	US	6051648
	US	6166130	US	6458889	US	6495127
	US	2001003126	US	2002042473	US	2003119985
	US	2003149173	WO	0116210	WO	9722371
	WO	02102864				
US 2001018614	AU	23445/00	AU	27861/01	AU	43150/01
	AU	57495/01	AU	64067/00	AU	92917/01
	CA	2350509	CA	2367376	CA	2397071
	CA	2399762	EP	1128849	EP	1181060
	EP	1246585	EP	1257306	EP	1333870
	US	6482584	US	6497726	US	6613278
	US	2001031254	US	2002072806	US	2002106393
	US	2002119437	US	2003023304	US	2003027125
	US	2003097179	WO	0029037	WO	0054821
	WO	0150999	WO	0178798	WO	0182993
	WO	0224244	WO	02064180	WO	03013342
	WO	03020107	WO	03051236		
US 5972385	AU	59203/98	EP	0994694	NZ	336480
	US	5866165	US	6309670	WO	9831345
US 2002183858	AU	40662/02	CA	2387300	EP	1264607
	JP	2003024340				

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ03/00116

US	2003039676	AU	37033/00	CA	2363153	EP	1152777
		JP	2002537073	US	6294187	US	2001043940
		WO	0050102				
JP	63229058						
FR	2687577						

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.